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BOTULINUM TOXIN SCREENING ASSAYS

CROSS REFERENCE TO RELATED APPLICATIONS

This is a national stage application under 35 U.S.C. § 371 of PCT application PCT/US2005/006421, filed on Feb. 23, 2005 which claims the benefit of provisional application Ser. No. 60/547,591 filed Feb. 24, 2004, which is hereby incorporated by reference in its entirety.

All of the publications cited in this application are hereby incorporated by reference herein in their entirety.

The myorelaxant properties of Botulinum toxins (BoNTs) are being exploited in a wide variety of therapeutic and cosmetic applications, see e.g., William J. Lipham, COSMETIC AND CLINICAL APPLICATIONS OF BOTULINUM TOXIN (Slack, Inc., 2004). For example, CoNTs therapies are proposed for treating dystonia, see e.g. Kei Roger Aoki, et al., Method for treating Dystonia with Botulinum Toxin C to G, U.S. Pat. No. 6,319,505 (Nov. 20, 2001); pain, see e.g., Kei Roger Aoki, et al., Method for Treating Pain by Peripheral Administration of a Neurotoxin, U.S. Pat. No. 6,464,986 (Oct. 15, 2002); muscle injuries, see e.g. Gregory F. Brooks, Methods for Treating Muscle Injuries, U.S. Pat. No. 6,423,319 (Jul. 23, 2002); cardiovascular diseases, see e.g. Gregory F. Brooks, Methods for Treating Cardiovascular Diseases with Botulinum Toxins, U.S. Patent Publication No. 2003/0185860 (Oct. 2, 2003); neuropsychiatric disorders, see e.g. Steven Donovan, Therapeutic Treatments for Neuropsychiatric Disorders, U.S. Patent Publication No. 2003/0211121 (Nov. 13, 2003); lower back pain, see e.g., Kei Roger Aoki, et al., Botulinum Toxin Therapy for Lower Back Pain, U.S. Patent Publication No. 2004/0037852 (Feb. 26, 2004); as well as other neuromuscular disorders, see e.g., Kei Roger Aoki, et al., Multiple Botulinum Toxins for Treating Neuromuscular Disorders and Conditions, U.S. Patent Publication No. 2001/0021695 (Sep. 13, 2001); Kei Roger Aoki, et al., Treatment of Neuromuscular Disorders and Conditions with Different Botulinum, U.S. Patent Publication No. 2002/0010138 (Jan. 24, 2002); Kei Roger Aoki, et al., Use of Botulinum Toxins for Treating Various Disorders and Conditions and Associated Pain, U.S. Patent Publication No. 2004/0013692 (Jan. 22, 2004). Additional proposed uses of BoNTs as biopharmaceutical neuro-modulators has expanded to cover a wide variety of treatments targeting certain disorders that lack a neuromuscular basis. For example, the effects on the autonomic nervous system has allowed the development of a Botulinum toxin serotype A (BoNT/A) therapy for treating axillary hyperhidrosis or sweating, and reports indicate BoNT/A may be an effective treatment for myofascial pain and tension, stroke, traumatic brain injury, cerebral palsy, gastrointestinal motility disorders, urinary incontinence cancer and migraine headaches. Lastly, cosmetic and other therapeutic applications are widely known. In fact, the expected use of BoNTs in both therapeutic and cosmetic treatments of humans is anticipated to expand to an ever widening range of diseases and ailments that can benefit from the myorelaxant properties of these toxins.

The growing clinical and therapeutic use of botulinum toxins necessitates the pharmaceutical industry to use accurate assays for BoNT activity in order to, for example, ensure accurate pharmaceutical formulations and monitor established quality control standards. In addition, given the potential danger associated with small quantities of BoNT in food-stuffs, the food industry requires BoNT activity assays, for example, to validate new food packaging methods and to ensure food safety. Additionally, BoNT activity assays are

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useful in identifying modulators of BoNT activity, for example, modulators that reduce BoNT activity which can be useful as a toxin antidote and modulators that increase BoNT activity which can be useful in creating more potent or longer lasting pharmaceutical formulations. The present invention provides novel BoNT assays for detecting the presence or activity of a BoNT useful for various industries, such as, e.g. the pharmaceutical and food industries, and provides related advantages as well.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a schematic of the current paradigm of the BoNT/A intoxication mechanism. This intoxication process can be described as comprising four steps: 1) receptor binding, where BoNT/A binds to a BoNT/A receptor system initiates the intoxication process; 2) complex internalization, where after BoNT/A binding, a vesicle containing a toxin/receptor system complex is endocytosed into the cell; 3) light chain translocation, where multiple events are thought to occur, including changes in the internal pH of the vesicle, formation of a channel pore comprising the H_N domain of BoNT/A heavy chain, separation of the BoNT/A light chain from the heavy chain, enzymatic activation of the light chain; and release of the activated light chain and 4) enzymatic target modification, where the activated light chain of BoNT/A proteolytically cleaves its target SNARE substrates, such as, e.g., SNAP-25.

FIG. 2 shows a schematic of an FGFR3 and the alternatively spliced exons that result in FGFR3IIb and FGFR3IIIc. The top diagram shows a generalized drawing of a FGFR3. The extracellular domain comprises a signal peptide (box labeled SP), three Ig-like domains (loops labeled IgI, IgII and IgIII) and an acid box (box labeled acid). A single membrane spanning region comprises the transmembrane domain (box labeled TM). The cytoplasmic portion of the receptor comprises the tyrosine kinase domain. The middle diagram shows a generalized drawing of the exons encoding a FGFR3IIb isoform, where exon 9 is spliced out from the primary transcript during processing. The lower diagram shows a generalized drawing of the exons encoding a FGFR3IIIc isoform, where exon 8 is spliced out from the primary transcript during processing.

FIG. 3 shows the results of electroporation of PURE-A into HIT-T15 cells. FIG. 3a shows the results of an inhibition of insulin release assay. The graph indicates that the addition of glucose to 25 mM induced insulin secretion from untreated cells (control) and cells subjected to electroporation without the addition of PURE-A (Electroporation No PURE-A). However, HIT-T15 cells into which PURE-A was introduced (Electroporation PURE-A) showed a decrease in insulin secretion indicating these cells were unresponsive to induction of insulin secretion. FIG. 3b shows the results of a SNAP-25 cleavage assay. Western blot analysis identified the presence of a BoNT/A SNAP-25₁₉₇ cleavage product in PURE-A treated cells (Electroporation PURE-A), but not in either control (Control and Electroporation No PURE-A), with equal amounts of protein loaded per lane and probed with an antibody that detects the BoNT/A SNAP-25₁₉₇ cleavage product.

FIG. 4 shows the affects of electroporation of HIT-T15 cells over time. FIG. 4a shows the results on an inhibition release for insulin assay demonstrating that the presence of the toxin delayed growth in HIT-T15 cells when compared to controls, but toxin-treated cells were able to replicate normally after a recovery period. FIG. 4b shows a western blot analysis demonstrating that cleavage of SNAP-25 was

TABLE 1

	FGFR Variants							
	FGFR1		FGFR2		FGFR3			
Variant	IIIb	IIIc	IIIb	IIIc	IIIb	IIIc	FGFR4	FGFR5
Ligands	FGF-1	FGF-1	FGF-1	FGF-1	FGF-1	FGF-1	FGF-1	FGF-1
	FGF-2	FGF-2	FGF-2	FGF-2	FGF-9	FGF-2	FGF-2	FGF-2
	FGF-3	FGF-4	FGF-7	FGF-4		FGF-4	FGF-4	
	FGF-8	FGF-5	FGF-10	FGF-5		FGF-8	FGF-6	
	FGF-10	FGF-6		FGF-6		FGF-9	FGF-8	
							FGF-9	
								FGF-9
								FGF-17
Tissues	Brain, bone, kidney, skin, lung, heart, muscle, neuron	Brain, kidney, skin, lung, liver, glial cells	Brain, CNS, kidney, skin, lung, testis	Lung, liver, kidney	Brain, skin, lung testis			

Table 1 - FGFR variants and ligand affinities. FGFR variants, associated ligands, and tissue distribution, see, e.g., Powers et al, supra, (2000); and Reuss & von Bohlen und Halbach, supra, (2003).

Diversity in FGF signaling beyond the five receptors is achieved in part by the generation of alternatively spliced variants encoding distinct receptor isoforms, see, e.g. Bernhard Hard Reuss & Oliver von Bohlen und Halbach, Fibroblast growth factors and their receptors in the central nervous system, 313(2) Cell Tissue Res. 139-157 (2003). The protein region that appears to have the highest influence on ligand binding specificity is a portion of the IgIII domain, for which isoforms encoded by three different splice variants have been identified. These three isoforms, designated IgIIIa, IgIIIb and IgIIIc, have relative binding affinities for different FGFR family members. Alternative splicing in the FGFR ligand binding domain, designated a and b, generates additional receptor isoforms with novel ligand affinities. Isoforms for IgIIIa, IgIIIb and IgIIIc have been identified for both FGFR1 and FGFR2. Thus far, the IgIIIa isoform of FGFR3 and the IgIIIa and IgIIIb isoforms of FGFR4 and FGFR5 have not been reported.

As mentioned above, FGFR3 commonly exists in two isoforms, FGFR3IIIc and FGFR3IIIb, which arise following alternative splicing of the primary transcript in which either exon 8 or 9 respectively is skipped (see FIG. 2). However, additional isoforms exist. For example, an FGFR3 isoform has been described which lacks the acid box, see, e.g., Akio Shimizu et al, A novel alternatively spliced fibroblast growth factor receptor 3 isoform lacking the acid box domain is expressed during chondrogenic differentiation of ATDC5 cells, 276(14) J. Biol. Chem. 11031-11040 (2001). In another example, a novel, potentially cytoplasmic isoform was recently identified, called FGFR3S, in which exons 8, 9 and 10 are spliced out creating a FGFR3 that lacks the second half of IgIIIc and the transmembrane domain, see, e.g., L-M. Sturla et al., FGFR3IIIS: a novel soluble FGFR3 spliced variant that modulates growth is frequently expressed in tumour cells, 89(7) Br. J. Cancer 1276-1284 (2003).

Aspects of the present invention provide, in part, a method of detecting BoNT/A activity by contacting a sample to a cell that contains an exogenous FGFR3 wherein said contacted cell is capable of BoNT/A intoxication and detecting the presence of BoNT/A activity of said contacted cell relative to a control cell, where a difference in said BoNT/A activity of said contacted cell as compared to said control cell is indicative of BoNT/A activity. In an embodiment, a method of detecting BoNT/A activity comprises contacting a sample to a cell that transiently contains an exogenous FGFR3 wherein said contacted cell is capable of BoNT/A intoxication and

detecting the presence of BoNT/A activity of said contacted cell relative to a control cell, where a difference in said BoNT/A activity of said contacted cell as compared to said control cell is indicative of BoNT/A activity. In another embodiment a method of detecting BoNT/A activity comprises contacting a sample to a cell that stably contains an exogenous FGFR3 wherein said contacted cell is capable of BoNT/A intoxication and detecting the presence of BoNT/A activity of said contacted cell relative to a control cell, where a difference in said BoNT/A activity of said contacted cell relative to a control cell as compared to said control cell is indicative of BoNT/A activity.

As used herein “botulinum toxin serotype A” is synonymous with “BoNT/A,” “type A,” or similar terminology referring unambiguously to *Clostridium botulinum* neurotoxin type A, means any of a number of polypeptide neurotoxins, and derivatives thereof, which can be purified from *Clostridium botulinum* serotype A strains and which share FGFR3 as a cell surface receptor. Such neurotoxins include those found in or corresponding to the following strains and accession numbers listed in Table 2.

TABLE 2

Strain	Accession No.
CL138	AAQ16535
137	AAQ16534
129	AAQ16533
13	AAQ16532
42N	AAQ16531
Hall A-hyper	AAM75961
667Ab	CAA61124
NCTC 2916	CAA36289
Allergan-Hall A	AAQ06331
62A	AAA23262
Kyoto-F	CAA51824
type A NIH	BAA11051
NCTC 7272	
7I03-H	
Kumgo	AAO21363

As used herein, the term “Fibroblast Growth Factor 3 Receptor” is synonymous with “FGFR3” and means a FGFR3 peptide or peptidomimetic which binds BoNT/A in a manner that elicits a BoNT/A intoxication response. FGFR3s useful in the invention encompass, without limitation, wild type FGFR3s, naturally occurring FGFR3 variants, non-naturally FGFR3 variants, such as, e.g., genetically engineered

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variants produced by random mutagenesis or rational designed, and active fragments derived from a FGFR3s. As a non-limiting example, a human FGFR3, naturally occurring human FGFR3 variants, non-naturally human FGFR3 variants, and human FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In another non-limiting example, a bovine FGFR3, naturally occurring bovine FGFR3 variants, non-naturally bovine FGFR3 variants, and bovine FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In another non-limiting example, a rat FGFR3, naturally occurring rat FGFR3 variants, non-naturally rat FGFR3 variants, and rat FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In still another non-limiting example, a mouse FGFR3, naturally occurring mouse FGFR3 variants, non-naturally mouse FGFR3 variants, and mouse FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In another non-limiting example, a chicken FGFR3, naturally occurring chicken FGFR3 variants, non-naturally chicken FGFR3 variants, and chicken FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In another non-limiting example, a frog FGFR3, naturally occurring frog FGFR3 variants, non-naturally frog FGFR3 variants, and frog FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In another non-limiting example, a newt FGFR3, naturally occurring newt FGFR3 variants, non-naturally newt FGFR3 variants, and newt FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. In another non-limiting example, a zebrafish FGFR3, naturally occurring zebrafish FGFR3 variants, non-naturally zebrafish FGFR3 variants, and zebrafish FGFR3 fragments that retain the ability to selectively bind BoNT/A and mediate the intoxication process, can be useful as a BoNT/A receptor in aspects of the present invention. It is also understood that both nucleic acid molecules, such as, e.g., DNA and RNA, that encode a FGFR3 disclosed in the present specification and peptide molecules or peptidomimetics comprising a FGFR3 disclosed in the present specification are useful in aspects of the present invention. SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 and 27 disclose nucleic acid molecules encoding representative of FGFR3s useful in aspects on the present invention, while SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26 and 28 disclose peptide molecules representative of FGFR3s useful in aspects on the present invention.

As used herein, the term "peptidomimetic" is used broadly to mean a peptide-like molecule that selectively binds BoNT/A as the peptide BoNT/A receptor upon which it is structurally based. Such peptidomimetics include chemically modified peptides, peptide-like molecules containing non-naturally occurring amino acids, and peptoids, which are peptide-like molecules resulting from oligomeric assembly of N-substituted glycines, and selectively bind BoNT/A as the peptide substrate upon which the peptidomimetic is derived, see, e.g. Goodman and Ro, Peptidomimetics for

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Drug Design, in "Burger's Medicinal Chemistry and Drug Discovery" Vol. 1 (ed. M. E. Wolff; John Wiley & Sons 1995), pages 803-861).

A variety of peptidomimetics are known in the art including, for example, peptide-like molecules which contain a constrained amino acid, a non-peptide component that mimics peptide secondary structure, or an amide bond isostere. A peptidomimetic that contains a constrained, non-naturally occurring amino acid can include, for example, an α -methylated amino acid; an α,α -dialkyl-glycine or α -aminocycloalkane carboxylic acid; an N^{α},C^{α} cyclized amino acid; an N -methylated amino acid; a β - or γ -amino cycloalkane carboxylic acid; an α,β -unsaturated amino acid; a β,β -dimethyl or β -methyl amino acid; a β -substituted-2,3-methano amino acid; an NC^{δ} or C^{α},C^{δ} cyclized amino acid; or a substituted proline or another amino acid mimetic. In addition, a peptidomimetic which mimics peptide secondary structure can contain, for example, a nonpeptidic β -turn mimic; γ -turn mimic; mimic of β -sheet structure; or mimic of helical structure, each of which is well known in the art. A peptidomimetic also can be a peptide-like molecule which contains, for example, an amide bond isostere such as a retro-inverso modification; reduced amide bond; methylenethioether or methylenesulfoxide bond; methylene ether bond; ethylene bond; thioamide bond; trans-olefin or fluoroolefin bond; 1,5-disubstituted tetrazole ring; ketomethylene or fluoroketomethylene bond or another amide isostere. One skilled in the art understands that these and other peptidomimetics are encompassed within the meaning of the term "peptidomimetic" as used herein.

Thus, in aspects of this embodiment, the FGFR3 can be a human FGFR3IIIB that selectively binds BoNT/A which has, e.g. at least 70% amino acid identity with the FGFR3 of SEQ ID NO: 2, at least 75% amino acid identity with the FGFR3 of SEQ ID NO: 2, at least 80% amino acid identity with the FGFR3 of SEQ ID NO: 2, at least 85% amino acid identity with the FGFR3 of SEQ ID NO: 2, at least 90% amino acid identity with the FGFR3 of SEQ ID NO: 2 or at least 95% amino acid identity with the FGFR3 of SEQ ID NO: 2. In other aspects of this embodiment, the FGFR3 is a human FGFR3IIIB that that selectively binds BoNT/A which has, e.g., at most one, two, three, four, five, six, seven, eight, nine, or ten amino acid substitutions relative to the FGFR3 of SEQ ID NO: 2.

In other aspects of this embodiment, the FGFR3 can be a human FGFR3IIIC that selectively binds BoNT/A which has, e.g. at least 70% amino acid identity with the FGFR3 of SEQ ID NO: 4, at least 75% amino acid identity with the FGFR3 of SEQ ID NO: 4, at least 80% amino acid identity with the FGFR3 of SEQ ID NO: 4, at least 85% amino acid identity with the FGFR3 of SEQ ID NO: 4, at least 90% amino acid identity with the FGFR3 of SEQ ID NO: 4 or at least 95% amino acid identity with the FGFR3 of SEQ ID NO: 4. In other aspects of this embodiment, the FGFR3 is a human FGFR3IIIC that that selectively binds BoNT/A which has, e.g., at most one, two, three, four, five, six, seven, eight, nine, or ten amino acid substitutions relative to the FGFR3 of SEQ ID NO: 4.

In other aspects of this embodiment, the FGFR3 can be a human FGFR3IIIS that selectively binds BoNT/A which has, e.g. at least 70% amino acid identity with the FGFR3 of SEQ ID NO: 6, at least 75% amino acid identity with the FGFR3 of SEQ ID NO: 6, at least 80% amino acid identity with the FGFR3 of SEQ ID NO: 6, at least 85% amino acid identity with the FGFR3 of SEQ ID NO: 6, at least 90% amino acid identity with the FGFR3 of SEQ ID NO: 6 or at least 95% amino acid identity with the FGFR3 of SEQ ID NO: 6. In

enzyme reactions, agarose gel electrophoresis, nucleic acid ligation, bacterial transformation, nucleic acid purification, nucleic acid sequencing are routine procedures well within the scope of one skilled in the art and from the teaching herein. Non-limiting examples of specific protocols necessary to make an expression construct are described in e.g. MOLECULAR CLONING A LABORATORY MANUAL, supra, (2001); and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Frederick M. Ausubel et al., eds. John Wiley & Sons, 2004). These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein.

A wide variety of expression vectors can be employed for expressing an open reading frame encoding a FGFR3 and include without limitation, viral expression vectors, prokaryotic expression vectors and eukaryotic expression vectors including yeast, insect and mammalian expression vectors. Non-limiting examples of expression vectors, along with well-established reagents and conditions for making and using an expression construct from such expression vectors are readily available from commercial vendors that include, without limitation, BD Biosciences-Clontech, Palo Alto, Calif.; BD Biosciences Pharmingen, San Diego, Calif.; Invitrogen, Inc., Carlsbad, Calif.; EMD Biosciences-Novagen, Madison, Wis.; QIAGEN, Inc., Valencia, Calif.; and Stratagene, La Jolla, Calif. The selection, making and use of an appropriate expression vector are routine procedures well within the scope of one skilled in the art and from the teachings herein.

It is envisioned that any of a variety of expression systems may be useful for expressing construct compositions disclosed in the present specification. An expression system encompasses both cell-based systems and cell-free expression systems. Cell-based systems include, without limited, viral expression systems, prokaryotic expression systems, yeast expression systems, baculoviral expression systems, insect expression systems and mammalian expression systems. Cell-free systems include, without limitation, wheat germ extracts, rabbit reticulocyte extracts and *E. coli* extracts. Expression using an expression system can include any of a variety of characteristics including, without limitation, inducible expression, non-inducible expression, constitutive expression, viral-mediated expression, stably-integrated expression, and transient expression. Expression systems that include well-characterized vectors, reagents, conditions and cells are well-established and are readily available from commercial vendors that include, without limitation, Ambion, Inc. Austin, Tex.; BD Biosciences-Clontech, Palo Alto, Calif.; BD Biosciences Pharmingen, San Diego, Calif.; Invitrogen, Inc., Carlsbad, Calif.; QIAGEN, Inc., Valencia, Calif.; Roche Applied Science, Indianapolis, Ind.; and Stratagene, La Jolla, Calif. Non-limiting examples on the selection and use of appropriate heterologous expression systems are described in e.g., PROTEIN EXPRESSION. A PRACTICAL APPROACH (S. J. Higgins and B. David Hames eds., Oxford University Press, 1999); Joseph M. Fernandez & James P. Hoeflner, GENE EXPRESSION SYSTEMS. USING NATURE FOR THE ART OF EXPRESSION (Academic Press, 1999); and Meena Rai & Harish Padh, *Expression Systems for Production of Heterologous Proteins*, 80(9) CURRENT SCIENCE 1121-1128, (2001). These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein.

An expression construct comprising a nucleic acid molecule encoding a FGFR3 disclosed in the present specification can be operationally-linked to a variety of regulatory elements that can positively or negatively modulate, either directly or indirectly, the expression of a nucleic acid molecule, such as, e.g. constitutive, tissue-specific, inducible or

synthetic promoters and enhancers. Non-limiting examples of constitutive regulatory elements include, e.g. the cytomegalovirus (CMV), herpes simplex virus thymidine kinase (HSV TK), simian virus 40 (SV40) early, 5' long terminal repeat (LTR), elongation factor-1 α (EF-1 α) and polyubiquitin (UbC) regulatory elements. Non-limiting examples of inducible regulatory elements useful in aspects of the present invention include, e.g., chemical-inducible regulatory elements such as, without limitation, alcohol-regulated, tetracycline-regulated, steroid-regulated, metal-regulated and pathogenesis-related; and physical-inducible regulatory elements such as, without limitation, temperature-regulated and light-regulated. Such inducible regulatory elements can be prepared and used by standard methods and are commercially available, including, without limitation, tetracycline-inducible and tetracycline-repressible elements such as, e.g. Tet-On™ and Tet-Off™ (BD Biosciences-Clontech, Palo Alto, Calif.) and the T-REX™ (Tetracycline-Regulated Expression) and Flp-In™ T-REX™ systems (Invitrogen, Inc., Carlsbad, Calif.); ecdisone-inducible regulatory elements such as, e.g. the CompleteControl™ Inducible Mammalian Expression System (Stratagene, Inc., La Jolla, Calif.); isopropyl β -D-galactopyranoside (IPTG)-inducible regulatory elements such as, e.g. the LacSwitch® II Inducible Mammalian Expression System (Stratagene, Inc., La Jolla, Calif.); and steroid-inducible regulatory elements such as, e.g. the chimeric progesterone receptor inducible system, GeneSwitch™ (Invitrogen, Inc., Carlsbad, Calif.). The skilled person understands that these and a variety of other constitutive and inducible regulatory systems are commercially available or well known in the art and can be useful in the invention for controlling expression of a nucleic acid molecule which encodes a BoNT/A receptor.

In an embodiment, a nucleic acid molecule encoding a FGFR3 can optionally be linked to a regulatory element such as a constitutive regulatory element. In aspects of this embodiment, a nucleic acid molecule encoding a mammalian FGFR3 can optionally be linked to a regulatory element such as a constitutive regulatory element; a nucleic acid molecule encoding a bird FGFR3 can optionally be linked to a regulatory element such as a constitutive regulatory element; a nucleic acid molecule encoding an amphibian FGFR3 can optionally be linked to a regulatory element such as a constitutive regulatory element; and a nucleic acid molecule encoding a fish FGFR3 can optionally be linked to a regulatory element such as a constitutive regulatory element.

In another embodiment, a nucleic acid molecule encoding a FGFR3 can optionally be linked to a regulatory element such as an inducible regulatory element. In aspects of this embodiment, a nucleic acid molecule encoding a mammalian FGFR3 can optionally be linked to a regulatory element such as a inducible regulatory element; a nucleic acid molecule encoding a bird FGFR3 can optionally be linked to a regulatory element such as an inducible regulatory element; a nucleic acid molecule encoding an amphibian FGFR3 can optionally be linked to a regulatory element such as an inducible regulatory element; and a nucleic acid molecule encoding a fish FGFR3 can optionally be linked to a regulatory element such as an inducible regulatory element. In another aspect of this embodiment, expression of the nucleic acid molecule is induced using, e.g., tetracycline-inducible, ecdisone-inducible or steroid-inducible.

It is understood that a FGFR3 useful in aspects of the present invention optionally can include one or more additional components. As a non-limiting example, a flexible spacer sequence such as poly-glycine sequences can be included in a FGFR3 useful in the invention. A useful FGFR3

can further include, without limitation, one or more of the following: epitope-binding tags, such as, e.g. FLAG, ExpressTM, human Influenza virus hemagglutinin (HA), human p62^{c-Myc} protein (c-MYC), Vesicular Stomatitis Virus Glycoprotein (VSV-G), glycoprotein-D precursor of Herpes simplex virus (HSV), V5, and AU1; affinity-binding, such as, e.g. polyhistidine (HIS), streptavidin binding peptide (strep), and biotin or a biotinylation sequence; peptide-binding regions, such as, e.g., the glutathione binding domain of glutathione-S-transferase, the calmodulin binding domain of the calmodulin binding protein, and the maltose binding domain of the maltose binding protein; immunoglobulin hinge region; an N-hydroxysuccinimide linker; a peptide or peptidomimetic hairpin turn; or a hydrophilic sequence or another component or sequence that, for example, promotes the solubility or stability of a FGFR3. Non-limiting examples of specific protocols for selecting, making and using an appropriate binding peptide are described in, e.g. Epitope Tagging, pp. 17.90-17.93 (Sambrook and Russell, eds., Molecular Cloning A Laboratory Manual, Vol. 3, 3rd ed. 2001); Antibodies: A Laboratory Manual (Edward Harlow & David Lane, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1998); and Using Antibodies: A Laboratory Manual: Portable Protocol No. I (Edward Harlow & David Lane, Cold Spring Harbor Laboratory Press, 1998). In addition, non-limiting examples of binding peptides as well as well-characterized reagents, conditions and protocols are readily available from commercial vendors that include, without limitation, BD Biosciences-Clontech, Palo Alto, Calif.; BD Biosciences Pharmingen, San Diego, Calif.; Invitrogen, Inc, Carlsbad, Calif.; QIAGEN, Inc., Valencia, Calif.; and Stratagene, La Jolla, Calif. These protocols are routine procedures well within the scope of one skilled in the art and from the teaching herein.

Aspects of the present invention provide, in part, a cell that contains an exogenous FGFR3 wherein said cell is capable of BoNT/A intoxication. As used herein, the term "cell," means any eukaryotic cell that expresses, or can be engineered to express, at least one exogenous FGFR3 that binds BoNT/A. The term cell encompasses cells from a variety of organisms, such as, e.g. murine, rat, porcine, bovine, equine, primate and human cells; from a variety of cell types such as, e.g. neural and non-neural; and can be isolated from or part of a heterogeneous cell population, tissue or organism. It is understood that cells useful in aspects of the invention can include, without limitation, primary cells; cultured cells; established cells; normal cells; transformed cells; tumor cells; infected cells; proliferating and terminally differentiated cells; and stably or transiently transfected cells, including stably and transiently transfected cells. It is further understood that cells useful in aspects of the invention can be in any state such as proliferating or quiescent; intact or permeabilized such as through chemical-mediated transfection such as, e.g. calcium phosphate-mediated, diethyl-laminoethyl (DEAE) dextran-mediated, lipid-mediated, polyethyleneimine (PEI)-mediated, polybrene-mediated, and protein delivery agents; physical-mediated ~~transfection~~ such as, e.g. biostatic particle delivery, microinjection and electroporation; and viral-mediated transfection, such as, e.g., retroviral-mediated transfection. It is further understood that cells useful in aspects of the invention may include those which express a FGFR3 under control of a constitutive, tissue-specific, cell-specific or inducible promoter element, enhancer element or both.

As used herein, the term "cell capable of BoNT/A intoxication" means a cell that can enable the overall cellular mechanism whereby BoNT/A proteolytically cleaves a substrate, such as, e.g. SNAP-25, and encompasses the binding of BoNT/A to a low or high affinity receptor, the internaliza-

tion of the toxin/receptor complex, the translocation of the BoNT/A light chain into the cytoplasm and the enzymatic target modification of a BoNT/A substrate. By definition, a cell capable of BoNT/A intoxication must express a FGFR3.

As a non-limiting example, a neuronal or non-neuronal cell can be transiently or stably engineered to express an exogenous nucleic acid molecule encoding a FGFR3. As another non-limiting example, a neuronal or non-neuronal cell can be transiently engineered to contain an exogenous FGFR3.

Cells useful in aspects of the invention include both neuronal and non-neuronal cells. Neuronal cells useful in aspects of the invention include, without limitation, primary neuronal cells; immortalized or established neuronal cells; transformed neuronal cells; neuronal tumor cells; stably and transiently transfected neuronal cells and further include, yet are not limited to, mammalian, murine, rat, primate and human neuronal cells. Non-limiting examples of neuronal cells useful in aspects of the invention include, e.g. peripheral neuronal cells, such as, e.g. motor neurons and sensory neurons; CNS neuronal cells, such as, e.g. spinal cord neurons like embryonic spinal cord neurons, dorsal root ganglia (DRG) neurons, cerebral cortex neurons, cerebellar neurons, hippocampal neurons and motor neurons. Neuronal cells useful in the invention can be, for example, central nervous system (CNS) neurons; neuroblastoma cells; motor neurons, hippocampal neurons or cerebellar neurons and further can be, without limitation, Neuro-2A, SH-SY5Y, NG108-15, N1E-115 or SK-N-DZ cells. The skilled person understands that these and additional primary and established neurons can be useful in the cells and methods of the invention.

Neurons useful in aspects of the invention include, without limitation, primary cultures such as primary cultures of embryonic dorsal root ganglion (DRG) neurons. As one example, primary cultures of embryonic rat DRG neurons are described in Mary J. Welch et al., Sensitivity of embryonic rat dorsal root ganglia neurons to *Clostridium botulinum* neurotoxins, 38(2) Toxicon 245 258 (2000); and primary cultures of fetal spinal cord neurons, for example, primary cultures of murine fetal spinal cord neurons are described in Elaine A. Neale et al., Botulinum neurotoxin A blocks synaptic vesicle exocytosis but not endocytosis at the nerve terminal, 147(6) J. Cell Biol. 1249-1260 (1999), and John A. Chaddock et al., Inhibition of vesicular secretion in both neuronal and non-neuronal cells by a retargeted endopeptidase derivative of *Clostridium botulinum* neurotoxin type A, 68(5) Infect. Immun. 2587-2593 (2000). Thus, in an embodiment, a cell capable of BoNT/A intoxication can be a neuron that contains an exogenous FGFR3. In aspects of this embodiment, a neuron can be a neuron from, e.g. a primary culture, an embryonic dorsal root ganglion primary culture or a fetal spinal cord primary culture. As non-limiting examples, cells useful according to a method disclosed in the present specification can include, a primary neuronal cell that contains an exogenous FGFR3, such as, e.g., a rat embryonic dorsal root ganglion (DRG) neuron that contains an exogenous FGFR3 or a murine fetal spinal cord neuron that contains an exogenous FGFR3.

Neuronal cell lines useful in aspects of the invention include, without limitation, neuroblastoma cell lines, neuronal hybrid cell lines, spinal cord cell lines, central nervous system cell lines, cerebral cortex cell lines, dorsal root ganglion cell lines, hippocampal cell lines and pheochromocytoma cell lines.

Neuroblastoma cell lines, such as, e.g. murine, rat, primate or human neuroblastoma cell lines can be useful in aspects of the invention. Neuroblastoma cell lines useful in aspects of the invention include, without limitation, BE(2)-C (ATCC

embryos can be immortalized, for example, by culturing the cells in conditioned media from a rat thyroid cell line that induces transformation in vitro. The immortalized cells can be differentiated into neurons expressing neuronal markers using the appropriate media; these differentiated cells express choline acetyltransferase and secrete acetylcholine and glutamate in response to depolarization and nicotine stimulation, see, e.g., David D. Allen et al., Impaired cholinergic function in cell lines derived from the cerebral cortex of normal and trisomy 16 mice, 12(9) Eur. J. Neurosci. 3259-3264 (2000). Thus, in an embodiment, a cell capable of BoNT/A intoxication can be a cerebral cortex cell that contains an exogenous FGFR3. In aspects of this embodiment, a cerebral cortex cell that contains an exogenous FGFR3 can be, e.g. a CNh cell that contains an exogenous FGFR3, HCN-1a cell that contains an exogenous FGFR3 and HCN-2 cell that contains an exogenous FGFR3.

Dorsal root ganglia cell lines, such as, e.g., murine, rat, primate and human dorsal root ganglia cell lines, can be useful in aspects of the invention and include, without limitation, G4b, see, e.g., David D. Allen et al., A dorsal root ganglia cell line derived from trisomy 16 fetal mice, a model for Down syndrome, 13(4) Neuroreport 491-496 (2002). Embryonic dorsal root ganglia primary cultures can be immortalized with transforming conditioned media as described above. Upon differentiation, the cell line exhibits neuronal traits and lacks glial markers by immunohistochemistry. Release of neurotransmitters such as acetylcholine can be induced in response to potassium and nicotine, see, e.g., Allen et al., supra, (2002). Thus, in an embodiment, a cell capable of BoNT/A intoxication can be a dorsal root ganglia cell that contains an exogenous FGFR3. In aspects of this embodiment, a dorsal root ganglia cell can be, e.g., a G4b cell that contains an exogenous FGFR3.

Hippocampal cell lines, such as, e.g. murine, rat, primate and human hippocampal lines can be useful in aspects of the invention and include, without limitation, HT-4, see, e.g., K. Frederiksen et al., Immortalization of precursor cells from the mammalian CNS, 1(6) Neuron 439-448 (1988) and HT-22, see, e.g. John B. Davis and Pamela Maher, Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line, 652(1) Brain Res. 169-173 (1994). As a non-limiting example, the murine hippocampal cell line HT-22 can be useful in the invention. As a further non-limiting example, the immortalized HN33 hippocampal cell line can be useful in the invention. This hippocampal cell line was derived from the fusion of primary neurons from the hippocampus of postnatal day 21 mice with the N18TG2 neuroblastoma cell line, and, when differentiated, shares membrane properties with adult hippocampal neurons in primary culture, see, e.g., Henry J. Lee et al., Neuronal Properties and Trophic Activities of Immortalized Hippocampal Cells from Embryonic and Young Adult Mice, 19(6) J. Neurosci. 1779-1787 (1990); and Henry J. Lee et al., Immortalized young adult neurons from the septal region: generation and characterization, 52(1-2) Brain Res. Dev Brain Res. 219-228 (1990). Thus, in an embodiment, a cell capable of BoNT/A intoxication can be a hippocampal cell that contains an exogenous FGFR3. In aspects of this embodiment, a hippocampal cell that contains an exogenous FGFR3 can be, e.g., a HT-4 cell that contains an exogenous FGFR3, a HT-22 cell that contains an exogenous FGFR3 and a HN33 cell that contains an exogenous FGFR3.

A variety of non-neuronal cells are useful in aspects of the invention. Non-neuronal cells useful in aspects of the invention include, without limitation, primary non-neuronal cells; immortalized or established non-neuronal cells; transformed

non-neuronal cells; non-neuronal tumor cells; stably and transiently transfected non-neuronal cells and further include, yet are not limited to, mammalian, murine, rat, primate and human non-neuronal cells. Non-neuronal cells useful in aspects of the invention further include, without limitation, any of the following primary or established cells: anterior pituitary cells; adrenal cells, such as, e.g., chromaffin cells of the adrenal medulla; pancreatic cells, such as, e.g., pancreatic acinar cells, pancreatic islet β cells and insulinoma HIT or INS-1 cells; ovarian cells, such as, e.g., steroid-producing ovarian cells; kidney cells, such as, e.g. inner medullary collecting duct (IMCD) cells; stomach cells, such as, e.g. enterochromaffin cells; blood cells, such as, e.g. erythrocytes, leucocytes, platelets, neutrophils, eosinophils, mast cells; epithelial cells, such as, e.g., those of the apical plasma membrane; fibroblasts; thyroid cells; chondrocytes; muscle cells; hepatocytes; glandular cells such as, e.g., pituitary cells, adrenal cells, chromaffin cells; and cells involved in glucose transporter (GLUT4) translocation. Thus, in an embodiment, 10 a cell capable of BoNT/A intoxication can be a non-neuronal cell. In aspects of this embodiment, a non-neuronal cell can be from a primary or established non-neuronal cell line from the, e.g. anterior pituitary cells, adrenal cells, pancreatic cells, ovarian cells, kidney cells, stomach cells, blood cells, epithelial cells, fibroblasts, thyroid cells, chondrocytes, muscle cells, hepatocytes and glandular cells.

As non-limiting examples, cells useful for detecting BoNT/A activity according to a method disclosed in the present specification can include, a primary or established 15 non-neuronal cell that contains an exogenous FGFR3, such as, e.g., a chromaffin cell that contains an exogenous FGFR3 or pancreatic acinar cell that contains an exogenous FGFR3; a primary neuronal cell that contains an exogenous FGFR3.

As discussed above, cells useful in the invention include 20 neuronal and non-neuronal cells that express low or undetectable levels of endogenous receptor but which have been transfected with, or otherwise engineered to express, one or more exogenous nucleic acid molecules encoding one or more FGFR3s. Cells useful in aspects of the present invention further include, without limitation, transformed, tumor or other cells which over-express one or more exogenous 25 FGFR3s. It is understood that the over-expressed receptor can be a wild type form of the receptor or can include one or more amino acid modifications as compared to the wild type receptor, with the proviso that the process of BoNT/A intoxication can still occur. As a non-limiting example, cells useful for detecting BoNT/A activity encompass those which express or over-express an exogenous mammalian FGFR3, such as, e.g. a human FGFR3, a bovine FGFR3, a rat FGFR3 or a mouse FGFR3. As another non-limiting example, cells useful for 30 detecting BoNT/A activity encompass those which express or over-express an exogenous bird FGFR3, such as, e.g. chicken FGFR3. As another non-limiting example, cells useful for detecting BoNT/A activity encompass those which express or over-express an exogenous amphibian FGFR3, such as, e.g., a newt FGFR3 or a frog FGFR3. As another non-limiting example, cells useful for detecting BoNT/A activity encompass those which express or over-express an exogenous fish FGFR3, such as, e.g. a zebrafish FGFR3.

Thus, in an embodiment, a cell capable of BoNT/A intoxication can be a cell stably expressing an exogenous FGFR3. In aspects of this embodiment, a cell capable of BoNT/A intoxication can be a cell stably expressing an exogenous mammalian FGFR3, such as, e.g. a human FGFR3, a bovine FGFR3, a rat FGFR3 or a mouse FGFR3. In other aspects of this embodiment, a cell capable of BoNT/A intoxication can be a cell stably expressing an exogenous bird FGFR3, such as,

e.g., chicken FGFR3. In other aspects of this embodiment, a cell capable of BoNT/A intoxication can be a cell stably expressing an exogenous amphibian FGFR3, such as, e.g., a newt FGFR3 or a frog FGFR3. In other aspects of this embodiment, a cell capable of BoNT/A intoxication can be a cell stably expressing an exogenous fish FGFR3, such as, e.g., a zebrafish FGFR3.

Aspects of the present invention provide, in part, detecting the presence of BoNT/A activity of said contacted cell relative to a control cell, where a difference in said BoNT/A activity of said contacted cell as compared to said control cell is indicative of BoNT/A activity. As used herein, the term "control cell" means a cell of the same or similar type as the contacted cell and grown under the same conditions but which is not contacted with any sample or is contacted with a defined negative sample or a defined positive sample. One skilled in the art understands that a variety of control cells are useful in the methods disclosed in the present specification and that a control cell can be a positive control cell or a negative control cell. A control cell can be, for example, a negative control cell such as a similar or identical cell containing the same or similar FGFR3 that is contacted with a similar, defined negative sample, which is known to lack active BoNT/A, or that is not contacted with any sample. A control cell also can be, for example, a positive control cell such as a similar or identical cell containing the same or similar FGFR3 contacted with a defined positive sample, which is known to include active BoNT/A.

A wide variety of assays can be used to determine the presence of BoNT/A activity, including direct and indirect assays for toxin uptake. Assays that determine BoNT/A binding or uptake properties can be used to assess BoNT/A activity. Such assays include, without limitation, cross-linking assays using labeled BoNT/A, such as, e.g., BoNT/A-SBED, see, e.g. Example II of the present specification and [125] BoNT/A, see, e.g., Noriko Yokosawa et al., Binding of *Clostridium botulinum* type C neurotoxin to different neuroblastoma cell lines, 57(1) Infect. Immun. 272-277 (1989); Noriko Yokosawa et al., Binding of botulinum type Cl, D and E neurotoxins to neuronal cell lines and synaptosomes, 29(2) Toxicon 261-264 (1991); and Tei-ichi Nishiki et al., Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes, 269(14) J. Biol. Chem. 10498-10503 (1994). Other non-limiting assays include immunocytochemical assays that detect toxin binding using labeled or unlabeled antibodies, see, e.g., Atsushi Nishikawa et al., The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells, 319(2) Biochem. Biophys. Res. Commun. 327-333 (2004) and immunoprecipitation assays, see, e.g. Yukako Fujinaga et al., Molecular characterization of binding sub-components of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes, 150(Pt 5) Microbiology 1529-1538 (2004). Antibodies useful for these assays include, without limitation, antibodies selected against a BoNT/A, antibodies selected against a BoNT/A receptor, such as, e.g., FGFR3, antibodies selected against a ganglioside, such as, e.g., GD1a, GD1b, GD3, GQ1b, or GT1b and selected against a test compound, such as, e.g., a molecule that selectively binds a BoNT/A receptor wherein selective binding modulates BoNT/A activity. If the antibody is labeled, the binding of the molecule can be detected by various means, including Western blotting, direct microscopic observation of the cellular location of the antibody, measurement of cell or substrate-bound antibody following a wash step, or electrophoresis, employing techniques well-known to those of skill in the art. If the antibody is unlabeled, one may

employ a labeled secondary antibody for indirect detection of the bound molecule, and detection can proceed as for a labeled antibody. It is understood that these and similar assays that determine BoNT/A uptake properties or characteristics can be useful in detecting BoNT/A activity.

Assays that monitor the release of a molecule after exposure to BoNT/A can also be used to assess for the presence of BoNT/A activity. In these assays, inhibition of the molecule's release would occur in cells expressing a FGFR3 after 10 BoNT/A treatment. As a non-limiting example the inhibition of insulin release assay disclosed in the present specification can monitor the release of a molecule after exposure to BoNT/A and thereby be useful in assessing whether a molecule selectively binds a BoNT/A receptor (see Example I). 15 Other non-limiting assays include methods that measure inhibition of radio-labeled catecholamine release from neurons, such as, e.g., [³H] noradrenaline or [³H] dopamine release, see e.g., A Fassio et al., Evidence for calcium-dependent vesicular transmitter release insensitive to tetanus toxin and 20 botulinum toxin type F, 90(3) Neuroscience 893-902 (1999); and Sara Stigliani et al., The sensitivity of catecholamine release to botulinum toxin Cl and E suggests selective targeting of vesicles set into the readily releasable pool, 85(2) J. Neurochem. 409-421 (2003), or measures catecholamine 25 release using a fluorometric procedure, see, e.g., Anton de Paiva et al., A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly, 268(28) J. Biol. 30 Chem. 20838-20844 (1993); Gary W. Lawrence et al., Distinct exocytotic responses of intact and permeabilised chromaffin cells after cleavage of the 25-kDa synaptosomal-associated protein (SNAP-25) or synaptobrevin by botulinum toxin A or B, 236(3) Eur. J. Biochem. 877-886 (1996); and 35 Patrick Foran et al., Botulinum neurotoxin [Cl] cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release, 35(8) Biochemistry 2630-2636 (1996); and methods that measure inhibition of hormone release from endocrine 40 cells, such as, e.g. anterior pituitary cells or ovarian cells. It is understood that these and similar assays for molecule release can be useful in assessing BoNT/A activity.

As non-limiting examples, an inhibition of insulin release assay can be used to determine the presence of BoNT/A 45 activity in cells containing a FGFR3 and capable of secreting insulin; an inhibition of noradrenaline release assay can be used to determine BoNT/A activity in cells containing a FGFR3 and capable of secreting noradrenaline; and an inhibition of estrogen release assay can be used to determine 50 BoNT/A activity in cells containing a FGFR3 and capable of secreting estrogen.

Assays that detect the cleavage of a BoNT/A substrate after exposure to BoNT/A can also be used to assess for the presence of BoNT/A activity. In these assays, generation of a 55 BoNT/A cleavage-product would be detected after BoNT/A treatment. As a non-limiting example the SNAP-25 cleavage assay disclosed in the present specification can detect the cleavage of a BoNT/A substrate after exposure to BoNT/A and thereby be useful in assessing BoNT/A activity (see 60 Example I). Other non-limiting methods useful to detect the cleavage of a BoNT/A substrate after exposure to BoNT/A are described in, e.g., Lance E. Steward et al., FRET Protease Assays for Botulinum Serotype A/E Toxins, U.S. Patent Publication No. 2003/0143650 (Jul. 31, 2003); and Ester [Femandez-Salas] et al., Cell-based Fluorescence Resonance Energy Transfer (FRET) Assays for Clostridial Toxins, U.S. Patent Publication 2004/0072270 (Apr. 15, 2004). It is understood 65

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that these and similar assays for BoNT/A substrate cleavage can be useful in assessing BoNT/A activity.

As non-limiting examples, western blot analysis using an antibody that recognizes BoNT/A SNAP-25-cleaved product can be used to determine the presence of BoNT/A activity. Examples of anti-SNAP-25 antibodies useful for these assays include, without limitation, rabbit polyclonal anti-SNAP25₁₉₇ antiserum pAb anti-SNAP25197 #1 (Allergan, Inc., Irvine, Calif.), mouse monoclonal anti-SNAP-25 antibody SMI-81 (Sternberger Monoclonals, Lutherville, Md.), mouse monoclonal anti-SNAP-25 antibody CI 71.1 (Synaptic Systems, Goettingen, Germany), mouse monoclonal anti-SNAP-25 antibody CI 71.2 (Synaptic Systems, Goettingen, Germany), mouse monoclonal anti-SNAP-25 antibody SP12 (Abcam, Cambridge, Mass.), rabbit polyclonal anti-SNAP-25 antiserum (Synaptic Systems, Goettingen, Germany), and rabbit polyclonal anti-SNAP-25 antiserum (Abcam, Cambridge, Mass.).

The methods disclosed in the present specification include, in part, a sample. As used herein, the term "sample" means any biological matter that contains or potentially contains an active BoNT/A. A variety of samples can be assayed according to a method disclosed in the present specification including, without limitation, purified, partially purified, or unpurified BoNT/A; recombinant single chain or di-chain toxin with a naturally or non-naturally occurring sequence; recombinant BoNT/A with a modified protease specificity; recombinant BoNT/A with an altered cell specificity; chimeric toxin containing structural elements from multiple BoNT/A species or subtypes; bulk BoNT/A; formulated BoNT/A product; and foods; cells or crude, fractionated or partially purified cell lysates, for example, engineered to include a recombinant nucleic acid encoding a BoNT/A; bacterial, baculoviral and yeast lysates; raw, cooked, partially cooked or processed foods; beverages; animal feed; soil samples; water samples; pond sediments; lotions; cosmetics; and clinical formulations. It is understood that the term sample encompasses tissue samples, including, without limitation, mammalian tissue samples, livestock tissue samples such as sheep, cow and pig tissue samples; primate tissue samples; and human tissue samples. Such samples encompass, without limitation, intestinal samples such as infant intestinal samples, tissue samples obtained from a wound. Other such samples include mammalian tissue, mammalian saliva, mammalian excretions and mammalian feces. As non-limiting examples, a method of the invention can be useful for detecting the presence or activity of a BoNT/A in a food or beverage sample; to assay a sample from a human or animal, for example, exposed to a BoNT/A or having one or more symptoms of a BoNT/A exposure; to follow activity during production and purification of BoNT/A; or to assay formulated BoNT/A products such as pharmaceuticals or cosmetics.

It is envisioned that a wide variety of processing formats can be used in conjunction with the methods disclosed present specification, including, without limitation, manual processing, partial automated-processing, semi-automated-processing, full automated-processing, high throughput processing, high content processing, and the like or any combination thereof.

Other aspect of the present invention provide methods of reducing BoNT/A activity in a human comprising administering to said human a pharmaceutical composition comprising a molecule that selectively binds a FGFR3 wherein said selective binding reduces the ability of BoNT/A to bind to said FGFR3. It is envisioned that any molecule that can selectively bind to a FGFR3 in a manner that prevents BoNT/A binding to that same FGFR3 can be useful, includ-

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ing, without limitation, an anti-FGFR3 antibody, an FGF or an FGF agonist. In addition, a FGFR3, a FGFR3 fragment retaining BoNT/A selective binding activity, or peptidomimetic thereof can also be useful. Molecules that selectively binds a FGFR3, and thus useful in methods of reducing BoNT/A activity are described in, e.g. Avner Yayon et al., Antibodies that block receptor protein tyrosine kinase activation, methods of screening for and using thereof, International Publication No. WO 02/102972 (Dec. 27, 2002); Avner 10 Yayon et al., Antibodies that block receptor protein tyrosine kinase activation, methods of screening for and using thereof, International Publication No. WO 02/102973 (Dec. 27, 2002); and Elisabeth Thomassen-Wolf et al., Antibodies that 15 block receptor protein tyrosine kinase activation, methods of screening for and using thereof, International Publication No. WO 02/102854 (Dec. 27, 2002) [REDACTED]

Aspects of the present invention provide, in part, a method of reducing BoNT/A activity in a human by administering a pharmaceutical composition comprising a molecule that 20 selectively binds a FGFR3. The administered composition can be formulated in a variety of pharmaceutically acceptable media, as described below. An effective dose of a composition disclosed in the present specification will depend upon the particular molecule selected, the route administration, and the 25 particular characteristics of the human or other mammal, such as age, weight, general health and the like. An effective dose can be determined in an animal model prior to administration to humans. Compositions useful in aspects of the invention can be administered by a variety of routes to stimulate an 30 immune response. As a non-limiting example, oral tolerance is well-recognized in the art (see, for example, Weiner, *Hospital Practice*, pp. 53-58 (Sep. 15, 1995). Those skilled in the art can readily determine for a particular composition, a suitable pharmacological composition, an appropriate antigen 35 payload; route of administration; volume of dose; and pharmaceutical regimen useful in a particular animal, for example, humans.

As disclosed herein a pharmaceutical composition is administered to a human or other mammal to reduce BoNT/A 40 activity. As used herein, the term "reduce," when used in reference to administering to a human or other mammal an effective amount of a pharmaceutical composition, means reducing a symptom of a condition characterized by exposure BoNT/A activity, or delaying or preventing onset of a symptom 45 of a condition characterized by exposure to BoNT/A activity in the human or other mammal. For example, the term "reducing" can mean reducing a symptom of a condition characterized by exposure to BoNT/A activity by at least 30%, 40%, 60%, 70%, 80%, 90% or 100%. The effectiveness 50 of a pharmaceutical composition in treating a condition characterized by exposure to BoNT/A activity can be determined by observing one or more clinical symptoms or physiological indicators associated with the condition. An improvement in a condition characterized by exposure to BoNT/A activity 55 also can be indicated by a reduced need for a concurrent therapy. Those of skill in the art will know the appropriate symptoms or indicators associated with specific conditions and will know how to determine if a human or other mammal is a candidate for treatment with a pharmaceutical composition disclosed in the present specification. In particular, it is understood that those skilled in the art will be able to determine if a condition is characterized by exposure BoNT/A activity, for example, by comparison of levels of BoNT/A activity from the human or other mammal with a normal control cells.

The appropriate effective amount to be administered for a particular application of the methods can be determined by

such as, e.g. neuronal cell lines including, without limitation, neuroblastoma cell lines, neuronal hybrid cell lines, spinal cord cell lines, central nervous system cell lines, cerebral cortex cell lines, dorsal root ganglion cell lines, hippocampal cell lines and pheochromocytoma cell lines. Non-limiting examples of neuronal cell lines include, e.g. neuroblastoma cell lines BE(2)-C, BE(2)-M17, C1300, CHP-212, CHP-126, IMR 32, KELLY, LA-N-2, MC-IXC, MHH-NB-11, N18Tg2, N1E-115, N4TG3, Neuro-2A, NB41A3, NS20Y, SH-SY5Y, SIMA, SK-N-DZ, SK-N-F1, SK-N-MC and SK-N-SH; neuroblastoma/glioma hybrid cell lines N18, NG108-15 and NG115-401L; neuroblastoma/motor neuron hybrid cell lines NSC-19 and NSC-32; neuroblastoma/root ganglion neuron hybrid cell lines F11, ND-E, ND-U1, ND7/23, ND8/34 and ND27; the neuroblastoma/hippocampal neuron hybrid cell line HN-33; spinal cord cell lines TE 189.T and M4b; cerebral cortex cell lines CNh, HCN-1a and HCN-2; dorsal root ganglia cell line G4b; hippocampal cell lines HT-4, HT-22 and HN33; FGFR3 expressing cell lines H929, JIM-3, KMS-11, KMS-18, LB278, LB375, LB1017, LB2100, LP-1, OPM-2, PCL1 and UTMC-2. In further aspects of this embodiment, an FGFR3 expressing cell can be, e.g., H929, JIM-3, KMS-11, KMS-18, LB278, LB375, LB1017, LB2100, LP-1, OPM-2, PCL1 UTMC-2, B9, TC, L6 and CFK2. Other aspects of this embodiment include cells, such as, e.g., non-neuronal cells including, without limitation, primary non-neuronal cells; immortalized or established non-neuronal cells; transformed non-neuronal cells; non-neuronal tumor cells; stably and transiently transfected non-neuronal cells expressing a FGFR3, and further include, yet are not limited to, mammalian, murine, rat, primate and human non-neuronal cells. Other aspects of this embodiment include cells, such as, e.g., non-neuronal cells useful in aspects of the invention further include, without limitation, anterior pituitary cells; adrenal cells, pancreatic cells, ovarian cells, kidney cells, stomach cell, blood cells, epithelial cells, fibroblasts, thyroid cells, chondrocytes, muscle cells, hepatocytes, glandular cells and cells involved in glucose transporter (GLUT4) translocation.

The molecule to be tested in the screening method may be a "small" organic compound of synthetic origin, or may be a macromolecule (either of synthetic or biological origin) including without limitation, a polypeptide, such as, e.g. a growth factor, a neurotoxin, a modified neurotoxin, an antibody or an antibody derivative; a nucleic acid, such as, e.g. a nucleic acid aptomer; and a polysaccharide, such as, e.g., a ganglioside or a lectin. In one embodiment, the molecule is a synthetic molecule designed based on the tertiary structure and three dimensional conformation of FGF or an antibody that inhibits BoNT/A binding to a FGFR3. Such SAR (structure/activity relationship) analysis is routine in the art of medicinal chemistry, among other fields.

A wide variety of assays can be used to determine whether a molecule selectively binds a FGFR3, including direct and indirect assays for toxin uptake. Assays that determine BoNT/A binding or uptake properties can be used to assess whether a molecule selectively binds a FGFR3. Such assays include, without limitation, cross-linking assays using labeled BoNT/A, such as, e.g., BoNT/A-SBED, see, e.g., Example II of the present specification and [¹²⁵I] BoNT/A, see, e.g. Noriko Yokosawa et al., Binding of *Clostridium botulinum* type C neurotoxin to different neuroblastoma cell lines, 57(1) Infect. Immun. 272-277 (1989); Noriko Yokosawa et al., Binding of botulinum type Cl, D and E neurotoxins to neuronal cell lines and synaptosomes, 29(2) Toxicon 261-264 (1991); and Tei-ichi Nishiki et al., Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes, 269(14) J. Biol. Chem. 10498-

10503 (1994). Other non-limiting assays include immunocytochemical assays that detect toxin binding using labeled or unlabeled antibodies, see, e.g. Atsushi Nishikawa et al., The receptor and transporter for internalization of *Clostridium botulinum* type C progenitor toxin into HT-29 cells, 319(2) Biochem. Biophys. Res. Commun. 327-333 (2004) and immunoprecipitation assays, see, e.g., Yukako Fujinaga et al., Molecular characterization of binding subcomponents of *Clostridium botulinum* type C progenitor toxin for intestinal epithelial cells and erythrocytes, 150(Pt 5) Microbiology 1529-1538 (2004). Antibodies useful for these assays include, without limitation, antibodies selected against a BoNT/A, antibodies selected against a BoNT/A receptor, such as, e.g., FGFR3, antibodies selected against a ganglioside, such as, e.g., GD1a, GD1b, GD3, GQ1b, or GT1b and selected against a test compound, such as, e.g., a molecule that selectively binds a BoNT/A receptor wherein selective binding modulates BoNT/A activity. If the antibody is labeled, the binding of the molecule can be detected by various means, including Western blotting, direct microscopic observation of the cellular location of the antibody, measurement of cell or substrate-bound antibody following a wash step, or electrophoresis, employing techniques well-known to those of skill in the art. If the antibody is unlabeled, one may employ a labeled secondary antibody for indirect detection of the bound molecule, and detection can proceed as for a labeled antibody. It is understood that these and similar assays that determine BoNT/A uptake properties or characteristics can be useful in selecting a neuron or other cells useful in aspects of the invention.

Assays that monitor the release of a molecule after exposure to BoNT/A can also be used to assess whether a molecule selectively binds a FGFR3. In these assays, inhibition of the molecule's release would occur in cells expressing a FGFR3 after BoNT/A treatment. As a non-limiting example the inhibition of insulin release assay disclosed in the present specification can monitor the release of a molecule after exposure to BoNT/A and thereby be useful in assessing whether a molecule selectively binds a FGFR3 (see Example I). Other non-limiting assays include methods that measure inhibition of radio-labeled catecholamine release from neurons, such as, e.g., [³H] noradrenaline or [³H] dopamine release, see e.g., A 40 Fassio et al., Evidence for calcium-dependent vesicular transmitter release insensitive to tetanus toxin and botulinum toxin type F, 90(3) Neuroscience 893-902 (1999); and Sara Stigliani et al., The sensitivity of catecholamine release to botulinum toxin Cl and E suggests selective targeting of vesicles set into the readily releasable pool, 85(2) J. Neurochem. 409-421 (2003), or measures catecholamine release using a fluorometric procedure, see, e.g., Anton de Paiva et al., A role for the interchain disulfide or its participating thiols in the internalization of botulinum neurotoxin A revealed by 45 a toxin derivative that binds to ecto-acceptors and inhibits transmitter release intracellularly, 268(28) J. Biol. Chem. 20838-20844 (1993); Gary W. Lawrence et al., Distinct exocytic responses of intact and permeabilised chromaffin cells after cleavage of the 25-kDa synaptosomal-associated protein (SNAP-25) or synaptobrevin by botulinum toxin A or B, 236(3) Eur. J. Biochem. 877-886 (1996); and Patrick Foran et al., Botulinum neurotoxin [Cl] cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release, 35(8) Biochemistry 2630-2636 (1996); and methods that measure inhibition of hormone release from endocrine cells, such as, e.g., anterior pituitary cells or ovarian cells. It is understood

that these and similar assays for molecule release can be useful in assessing whether a molecule selectively binds a FGFR3.

As non-limiting examples, an inhibition of insulin release assay can be used to test whether a molecule selectively binds a FGFR3 in a FGFR3 containing cells capable of secreting insulin; an inhibition of noradrenaline release assay using can be used to test whether a molecule selectively binds a FGFR3 in a FGFR3 containing cells capable of secreting noradrenaline; and an inhibition of estrogen release assay can be used to assay whether a molecule selectively binds a FGFR3 in a FGFR3 containing cells and capable of secreting estrogen.

Assays that detect the cleavage of a BoNT/A substrate after exposure to BoNT/A can also be used to assess whether a molecule selectively binds a FGFR3. In these assays, generation of a BoNT/A cleavage-product would be detected in cells expressing a FGFR3 after BoNT/A treatment. As a non-limiting example the SNAP-25 cleavage assay disclosed in the present specification can detect the cleavage of a BoNT/A substrate after exposure to BoNT/A and thereby be useful in assessing whether a molecule selectively binds a BoNT/A receptor (see Example I). Other non-limiting methods useful to detect the cleavage of a BoNT/A substrate after exposure to BoNT/A are described in, e.g. Lance E. Steward et al., FRET Protease Assays for Botulinum Serotype A/E Toxins, U.S. Patent Publication No. 2003/0143650 (Jul. 31, 2003); and Ester Fernandez-Salas et al., Cell-based Fluorescence Resonance Energy Transfer (FRET) Assays for Clostridial Toxins, U.S. Patent Publication 2004/0072270 (Apr. 15, 2004). It is understood that these and similar assays for BoNT/A substrate cleavage can be useful in assessing whether a molecule selectively binds a FGFR3.

As non-limiting examples, western blot analysis using an antibody that recognizes BoNT/A SNAP-25-cleaved product can be used to assay whether a molecule selectively binds a FGFR3. Examples of anti-SNAP-25 antibodies useful for these assays include, without limitation, rabbit polyclonal anti-SNAP25₁₉₇ antiserum pAb anti-SNAP25197 #1 (Allergan, Inc., Irvine, Calif.), mouse monoclonal anti-SNAP-25 antibody SMI-81 (Sternberger Monoclonals, Lutherville, Md.), mouse monoclonal anti-SNAP-25 antibody CI 71.1 (Synaptic Systems, Goettingen, Germany), mouse monoclonal anti-SNAP-25 antibody CI 71.2 (Synaptic Systems, Goettingen, Germany), mouse monoclonal anti-SNAP-25 antibody SP12 (Abcam, Cambridge, Mass.), rabbit polyclonal anti-SNAP-25 antiserum (Synaptic Systems, Goettingen, Germany), and rabbit polyclonal anti-SNAP-25 antiserum (Abcam, Cambridge, Mass.).

Assays that detect competitive binding of a molecule with BoNT/A for selective binding to a FGFR3 can also be used to assess whether a molecule selectively binds a FGFR3. In these assays, a reduction in BoNT/A activity would be detected as the amount of a molecule that competes with BoNT/A for selective binding to a BoNT/A would increase. In a non-limiting example, the competitive inhibition assay using FGF ligands disclosed in the present specification can be used to detect the competitive binding of a molecule with BoNT/A for selective binding to a FGFR3 and thereby be useful in assessing whether a molecule selectively binds a BoNT/A receptor (see Example II). Thus in one aspect of this embodiment, competitive binding assays using a FGFR3-binding molecule with BoNT/A for selective binding to a FGFR3 can be used to assess whether a molecule selectively binds a FGFR3.

Other aspect of the present invention provide methods of rendering a cell susceptible to cleavage of SNARE proteins by BoNT/A, comprising inducing said cell to express a

FGFR3. Other aspect of the present invention provide methods of transiently rendering a cell susceptible to cleavage of SNARE proteins by BoNT/A, comprising transiently inducing said cell to express a FGFR3. Other aspect of the present invention provide methods of stably rendering a cell susceptible to cleavage of SNARE proteins by BoNT/A, comprising stably inducing said cell to express a FGFR3.

Other aspect of the present invention provide methods of marketing a neurotoxin capable of selectively binding to the same FGFR3 as BoNT/A comprising obtaining marketing approval from a governmental or regional regulatory authority for a therapeutic neurotoxin, wherein said neurotoxin is assayed for selective binding to a cell comprising contacting said neurotoxin with a composition comprising a FGFR3 and detecting whether said neurotoxin selectively binds said FGFR3, wherein selective binding of said neurotoxin to said FGFR3 indicates that said neurotoxin is able to selective binding to cells susceptible to BoNT/A intoxication and wherein if said molecule is BoNT/A, said method does not comprise an LD₅₀ assay; packaging said neurotoxin for sale in a manner consistent with the requirements of said regulatory authority, and selling said neurotoxin.

Other aspect of the present invention provide methods of marketing a neurotoxin capable of selectively binding to the same FGFR3 as BoNT/A comprising obtaining marketing approval from a governmental or regional regulatory authority for a therapeutic neurotoxin, wherein said neurotoxin is assayed for selective binding to a cell comprising contacting said neurotoxin to a cell that contains an exogenous FGFR3 wherein said contacted cell is capable of BoNT/A intoxication and detecting the presence of BoNT/A activity of said contacted cell relative to a control cell, where a difference in said BoNT/A activity of said contacted cell as compared to said control cell is indicative of BoNT/A activity; packaging said neurotoxin for sale in a manner consistent with the requirements of said regulatory authority, and selling said neurotoxin.

In another embodiment, the invention is drawn to a polypeptide comprising at least the H_C region of BONT/A, which is produced from a bulk or formulated preparation wherein the bulk or formulated preparation is assayed for specific binding to neural cells using a method comprising contacting said polypeptide with a composition comprising FGFR3 receptor and, optionally, GT1b ganglioside, and detecting whether said polypeptide selectively binds FGFR3.

In another embodiment similar to the above aspect of the invention, the polypeptide comprises at least an FGFR3 binding domain, other than the H_C domain of BoNT/A. Such a binding domain may comprise, for example, an FGF, such as FGF 1, FGF2, FGF4, FGF8 or FGF 9, or an anti-FGFR3 antibody. Further, the polypeptide may optionally contain a translocation domain such as the H_N domain of BoNT/A. Additionally, the polypeptide will generally contain a clostridial neurotoxin light chain or variation thereof—the nature and/or source of the light chain can provide differences in the extent and half-life of the therapeutic effect of the polypeptide.

Thus, in this embodiment the claimed polypeptide is produced (which production may include purification, enzymatic treatment, and/or oxidation steps) from a bulk or formulation preparation. In one embodiment the preparation may be, for example, a cell lysate from fermentation of a BoNT/A-producing strain of *Clostridium botulinum*, or from a suitable mammalian, insect or bacterial host cell producing a recombinant version of BoNT/A. Such a bulk preparation may also be produced using cell-free transcription methodologies. In another embodiment the preparation may be puri-

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fied BoNT/A formulated with associated stabilizing proteins, such as serum albumin. In each case, the preparation may comprise BoNT/A molecules which are denatured or otherwise incorrectly folded so as not to bind to the target cells. The potency and/or specific activity of the preparation, or of fractions purified from the preparation, can be detected by using the claimed assay method.

Alternatively, the polypeptide to be assayed may comprise only a portion of the entire BoNT/A molecule. For example, the bulk preparation may contain only the heavy chain of BoNT/A, as separate production of the heavy and light chains of the toxin may be a preferred way of avoiding accidental exposure to the neurotoxin by laboratory workers.

As another example of the above embodiment, the polypeptide may comprise a chimeric recombinant polypeptide which contains the H_C region of the heavy chain of BoNT/A (or some other FGFR3-binding moiety, such as FGF itself). The chimeric polypeptide comprises amino acid sequence regions additional to, or other than, those present in the wild-type BoNT/A BoNT/A molecule. For example, botulinum and tetanus toxins may be used as the basis for the creation of transport proteins, see, e.g., James Oliver Dolly et al., Modification of clostridial toxins for use as transport proteins, U.S. Pat. No. 6,203,794 (Mar. 20, 2001). The light chain of these transport proteins are generally either replaced by a therapeutic moiety or inactivated and coupled to such a therapeutic moiety. Additionally, chimeric neurotoxins can be made comprising polypeptides containing domains of more than one neurotoxin see, e.g., James Oliver Dolly et al., Activatable Recombinant Neurotoxins, International Publication No. WO 01/14570 (Mar. 1, 2001). Thus, this aspect of the invention also encompasses, as a embodiment, chimeric neurotoxins containing at least the H_C domain of BoNT/A. Such molecules may be useful in modulating the time or extent of the inhibition of secretory vesicle release. Further, it may be desirable to target agents, such as therapeutic agents, to the extracellular surface of the neural cell membrane. Thus, such an agent may be joined (e.g., as a fusion protein or via post translational conjugation) to the H_C portion of BoNT/A. In such a case the cell lysate or conjugation reaction mixture may comprise a batch preparation in accordance with this aspect of the invention.

The above-referenced polypeptides are screened for binding and/or internalization essentially as mentioned above in the described screening method embodiment.

In yet another embodiment, the present invention is drawn to a method of marketing a polypeptide which contains a region capable of binding the FGFR3 receptor comprising obtaining permission from a governmental or regional drug regulatory authority to sell said polypeptide, wherein said polypeptide is first produced from a bulk preparation which is assayed for selective binding of said polypeptide to neural cells by contacting the bulk preparation containing said polypeptide with a composition comprising FGFR3 receptor, and optionally GT1b ganglioside, and detecting whether said polypeptide selectively binds FGFR3 under such conditions, packaging said polypeptide for sale in a manner consistent with the requirements of said regulatory authority, and offering said polypeptide for sale.

In this embodiment the invention is drawn to a method of marketing a polypeptide containing the H_C region of a BoNT/A toxin. The polypeptide at issue in this embodiment of the invention is produced from a bulk preparation which is assayed for purity or activity using the screening method described previously. In a step of this method, permission is obtained from a regulatory body for the marketing of such polypeptide. In this context "permission" may be tacit or

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express; that is, permission or approval may be obtained from the regulatory authority for the sale of a therapeutic agent or composition comprising said polypeptide, in which case "permission" is marketing approval for the sale of such agent or composition. Alternatively, "permission", as used herein, may comprise the assent, either affirmatively given or manifested by its lack of objection, of such regulatory authority to the continued sale of a product containing a polypeptide assayed in this new manner. As before, the polypeptide may comprise BoNT/A, or a derivative thereof, or a fusion protein or conjugate containing the H_C region of the BoNT/A heavy chain.

The therapeutic product comprising the polypeptide originally contained in the bulk preparation so assayed is labeled in accordance with the requirements of the regulatory authority. The product is then offered for sale. Offering for sale may comprise advertising or sales activity, educational seminars directed at doctors, hospitals, insurers, or patients, conversations with state, regional or governmental officials concerning subsidy reimbursement (such as Medicare or Medical).

EXAMPLES

Example I

Identification of a BoNT/A Receptor Using a Genetic Complementation Procedure

1. Identification of Cells Useful in Screening for a BoNT/A Receptor

1a. Identification of BoNT/A Receptor Lacking Cells Using an Inhibition Assay for Insulin Release

To determine whether HIT-T15 cells express a receptor for BoNT/A, an inhibition assay for insulin release was performed. In response to glucose stimulation, the hamster insulinoma cell line HIT-T15 secretes insulin in a exocytic process that depends on the activity of SNAP-25 for vesicle docking and fusion. If HIT-T15 cells lack a BoNT/A receptor, these cells would be unable to uptake BoNT/A upon exposure to this toxin and insulin secretion could occur in the presence of high glucose in the media. However, if HIT-T15 cells contain a BoNT/A receptor, insulin secretion would be inhibited after BoNT/A treatment since the toxin could intoxicate the cell and cleave SNAP-25.

To conduct an inhibition assay for insulin release, a suitable seed density of approximately 1.5×10^5 cells/mL of HIT-T15 cells was plated into individual wells of 6-well, poly-D-lysine/Laminin coated, tissue culture plates containing 3 mL of complete Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 1x penicillin/streptomycin solution (Invitrogen, Inc, Carlsbad, Calif.) and 4 mM Glutamine (Invitrogen, Inc, Carlsbad, Calif.), and grown in a 37° C. incubator under 5% carbon dioxide until the cells reach a density of about 5×10^5 cells/ml (6-16 hours). A group of HIT-T15 cells were treated with approximately 1 nM of PURE-A by introducing the toxin using electroporation using a GENE PULSER® II set at 960 μ F and 0.28 kV (Bio-Rad Laboratories, Hercules, Calif.). An untreated control group underwent electroporation without PURE-A. The media from the wells containing treated and untreated electroporated cells was replaced with 3 mL of fresh complete DMEM supplement with either 5.6 mM glucose (low glucose) or 25 mM glucose (high glucose) and these cells were incubated in a 37° C. incubator under 5% carbon dioxide for approximately 1 hour to induce insulin secretion. The conditioned media was transferred to 15 mL tubes and

the amount of insulin present in the condition media samples was determined using an Insulin ELISA assay (Peninsula Laboratories, Inc., San Carlos, Calif.). Exocytosis is expressed as the amount of insulin secreted per 1.5×10^5 cell/hr. Insulin release was detected in BoNT/A-untreated cells simulated by 25 mM glucose, but insulin secretion was inhibited in BoNT/A-treated cells (see FIG. 3a). These data indicate that the release of insulin in HIT-T15 cells is mediated, in part, by SNAP-25, but that these cells lack a BoNT/A receptor.

1b. Identification of BoNT/A Receptor Lacking Cells Using an Using a SNAP-25 Cleavage Assay

To determine whether HIT-T15 cells express a receptor for BoNT/A, a SNAP-25 cleavage assay was performed. If HIT-T15 cells lack a BoNT/A receptor, then only the presence of the uncleaved SNAP-25 substrate would be detected after Western blot analysis. However, if HIT-T15 cells contain a BoNT/A receptor, then the toxin could intoxicate the cell and the presence of the cleaved BoNT/A SNAP-25₁₉₇ product would be detected.

To conduct a SNAP-25 cleavage assay, cells were grown in poly-D-lysine/Laminin coated 6-well plates and treated with PURE-A as described above in Example I, 1a. Cells were collected in 15 ml tubes, washed once with 1 ml of phosphate-buffered saline, pH 7.4, and then transferred to 1.5 ml microcentrifuge tubes. Cells were lysed in 0.5 ml of lysis buffer containing 50 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 6.8, 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10% glycerol and 1% (v/v) Triton-X® 100 (4-octylphenol polyethoxylate), with rotation for 1 hour at 4° C. Lysed cells were centrifuged at 5000 rpm for 10 min at 4° C. to eliminate debris and the supernatants were transferred to fresh siliconized tubes. Protein concentrations were measured by Bradford's method and resuspended in 1×SDS sample buffer at 1 mg/mL or higher concentration.

To detect for the presence of a cleaved BoNT/A substrate, samples were boiled for 5 min, and 40 µl aliquots were separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen, Inc, Carlsbad, Calif.) under denaturing, reducing conditions. Separated peptides were transferred from the gel onto polyvinylidene fluoride (PVDF) membranes (Invitrogen, Inc, Carlsbad, Calif.) by Western blotting using a Trans-Blot® SD semi-dry electrophoretic transfer cell apparatus (Bio-Rad Laboratories, Hercules, Calif.). PVDF membranes were blocked by incubating at room temperature for 2 hours in a solution containing 25 mM Tris-Buffered Saline (25 mM 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloric acid (Tris-HCl) (pH 7.4), 137 mM sodium chloride, 2.7 mM potassium chloride), 0.1% TWEEN-20, polyoxyethylene (20) sorbitan monolaurate, 2% bovine serum albumin, 5% nonfat dry milk. Blocked membranes were incubated at 4° C. for overnight in Tris-Buffered Saline TWEEN-20® (25 mM Tris-Buffered Saline, 0.1% TWEEN-20', polyoxyethylene (20) sorbitan monolaurate) containing a 1:5,000 dilution of rabbit polyclonal anti-SNAP25 antiserum pAb anti-SNAP25 197 #1, a polyclonal antibody which is specific for the SNAP25₁₉₇-cleavage product and does not cross-react with full-length SNAP25₂₀₆, (Allergan, Inc., generated under contract with Zymed Laboratories Inc., South San Francisco, Calif.). Primary antibody probed blots were washed three times for 15 minutes each time in Tris-Buffered Saline TWEEN-20. Washed membranes were incubated at room temperature for 2 hours in

Tris-Buffered Saline TWEEN-20® containing a 1:20,000 dilution of goat polyclonal anti-rabbit immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (HRP; Pierce Biotechnology, Inc., Rockford, Ill.) as a secondary antibody. Secondary antibody-probed blots were washed three times for 15 minutes each time in Tris-Buffered Saline TWEEN-20®. Signal detection of the labeled BoNT/A SNAP25₁₉₇-cleavage product was visualized using the ECL Plus™ Western Blot Detection System (Amersham Biosciences, Piscataway, N.J.) and the membrane was imaged and cleavage product quantitated with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (Amersham Biosciences, Piscataway, N.J.). The choice of pixel size (100 to 200 pixels) and PMT voltage settings (350 to 600, normally 400) depended on the individual blot. A BoNT/A SNAP25₁₉₇-cleavage product was detected in HIT-T15 cell treated with BoNT/A but not untreated cells, indicating that HIT-T15 cells express SNAP-25 but not the BoNT/A receptor (see FIG. 3b).

20 1c. Assessment of BoNT/A Exposure on HIT-T15 Growth
To evaluate if the presence of the toxin in the cells affect cell growth, HIT-T15 cells were electroporated as described above in Example I, 1a and monitored for 10 days. FIG. 4a demonstrates that the presence of the toxin delayed growth when compared to controls, but toxin-treated cells were able to replicate normally after a recovery period. Cell aliquots for days 3, 5, 7 and 10 were also tested for the presence of the BoNT/A SNAP-25₁₉₇ cleavage product using the SNAP-25 cleavage assay as described above in Example I, 1b. FIG. 4b shows that cleavage of SNAP-25 was detected by Western blot analysis at all time points assayed when PURE-A was introduced into the cells.

25 35 2. Identification of BoNT/A Receptor Using Genetic Complementation

To identify a BoNT/A receptor, a nucleic acid molecule encoding a BoNT/A receptor was cloned by genetic complementation. This procedure involves introducing a nucleic acid molecule encoding the BoNT/A receptor into a cell line that does not contain the receptor naturally by retroviral transduction, see, e.g. Mitchell H. Finer et al., Methods for Production of High Titer Virus and High Efficiency Retroviral Mediated Transduction of Mammalian Cells, U.S. Pat. No. 5,858,740 (Jul. 12, 1999).

40 45 50 55 60 65 2a. Production of a Retroviral Stock Containing pLIB Expression Constructs

To produce an retroviral stock containing expression constructs encoding human brain nucleic acid molecules, about 5×10^5 HEK 293-based cells (AmphoPack™ 293 cells; BD Biosciences Clontech, Palo Alto, Calif.) were plated in 60 mm tissue culture dishes containing 5 mL of complete Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 1× penicillin/streptomycin solution (Invitrogen, Inc, Carlsbad, Calif.) and 4 mM Glutamine (Invitrogen, Inc, Carlsbad, Calif.), and grown in a 37° C. incubator under 5% carbon dioxide until the cells reach 60% to 80% confluence or a density of about 1 to 2×10^6 cells/ml (12-24 hours). On the day of transfection, the complete, supplemented DMEM media was replaced with 3 mL of OPTI-MEM Reduced Serum Medium. A 500 µL transfection solution is prepared by adding 250 µL of OPTI-MEM Reduced Serum Medium containing 15 µL of LipofectAmine 2000 (Invitrogen, Carlsbad, Calif.) incubated at room temperature for 5 minutes to 250 µL of OPTI-MEM Reduced Serum Medium containing 5 µg of pLIB retroviral expression constructs containing nucleic acid molecules derived from

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human brain cells (BD Biosciences Clontech, Palo Alto, Calif.). This transfection is incubated at room temperature for approximately 20 minutes. The 500 μ L transfection solution was then added to the AmphiPackTM 293 cells and the cells were incubated in a 37° C. incubator under 5% carbon dioxide for approximately 8-10 hours. The transfection media was replaced with 3 mL of fresh complete, supplemented DMEM and cells were incubated in a 37° C. incubator under 5% carbon dioxide for approximately 48-72 hours. The retrovirus-containing cells are harvested by detaching the cells using the culture media and scraping cells from the culture plate. Detached cells and media are transferred to a 15 mL tube and centrifuged (5,000 \times g at 20° C. for 15 minutes) to pellet the cellular debris. The clarified supernatant containing the retroviral particles is transferred to 2 mL cryovials in 1 mL aliquots and should contain approximately 5 \times 10⁴ to 5 \times 10⁶ tu/mL of retroviral particles. Aliquots can be stored at -80° C. until needed.

2b. Transduction of Cells with a Retroviral Stock Containing pLIB Expression Constructs

To transduce cells with a retroviral stock containing expression constructs encoding human brain nucleic acid molecules, about 1.5 \times 10⁵ HIT-T15 cells were plated in 60 mm tissue culture dishes containing 5 mL of complete Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 1x penicillin/streptomycin solution (Invitrogen, Inc., Carlsbad, Calif.) and 4 mM Glutamine (Invitrogen, Inc., Carlsbad, Calif.), and grown in a 37° C. incubator under 5% carbon dioxide until the cells reach 60% to 80% confluence or a density of about 5 \times 10⁵ cells/mL (6-16 hours). Cells are inoculated with the retroviral stock containing nucleic acid molecules derived from human brain cells (see Example I, 2a), using a suitable multiplicity of infection. Approximately 4-8 μ g/mL of polybrene was then added and the cells were incubated for approximately 16-24 hours in a 37° C. incubator under 5% carbon dioxide. The transduction media is replaced with 5 mL of fresh complete, supplemented DMEM and the cells were incubated in a 37° C. incubator under 5% carbon dioxide for approximately four days. The transduced cells were then used to conduct a screening assay to identify a BoNT/A receptor. For greater details on procedures described in this example, see Retroviral Gene Transfer and Expression User Manual PT3132-1 (PR43789), BD Biosciences Clontech, Palo Alto, Calif., (Mar. 3, 2004).

2c. Screening of HIT-T15 Cells Expressing a Retroviral cDNA Library

To screen for cells expressing a BoNT/A receptor, transduced HIT-T15 cells as described above in Example I, 2b were screened based on their ability to bind Dynex Beads coated with Pure A (ref). Approximately 7.5 mg of Dynabeads[®] magnetic beads (Dynal Biotechnology, LLC, Brown Deer, Wis.) coated with an antibody against the light chain of BoNT/A was added to the media for 30 minutes at 4° C. and cells binding to the BoNT/A light chain were isolated as clumps of cells after exposure to a magnet. These isolated cells were washed once with PBS and transferred to new 60 mm tissue culture dishes containing 5 mL of complete DMEM. These cells were re-screened with 7.5 mg of Dynabeads[®] magnetic beads coated with PURE-A for 30 minutes at 4° C. and cells binding to PURE-A were isolated as clumps of cells after exposure to a magnet (see FIG. 5). These re-isolated cell colonies were transferred to 96-well plates containing 0.25 mL of complete DMEM and the cells were grown in a 37° C. incubator under 5% carbon dioxide until confluent.

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To test for the presence of a BoNT/A receptor, individual, cells contained in the 96-well plates were assayed using the inhibition assay for insulin release assay, as described above in Example I, 1a. Cell lines containing a candidate BoNT/A receptor were selected based on the detection of the inhibition of insulin release. FIG. 6 show that transduced HIT-T15 cell lines C6 and C7 as candidate cell lines expressing a BoNT/A receptor. To confirm these results, expanded cultures of clones C6 and C7 as described above in Example I, 2a and tested using the inhibition of insulin release assay and the SNAP-25 cleavage assay, as described above in Example I, 1b. The results indicate that a BoNT/A receptor is present in these cell lines based on the inhibition of insulin release (see FIG. 7a) and the presence of a BoNT/A SNAP25₁₉₇-cleavage product (see FIG. 7b).

2d. Cloning of BoNT/A Receptor

To isolate nucleic acid molecules encoding the BoNT/A receptor, DNA will be purified from the BoNT/A receptor-containing HIT-T15 cell isolates identified above in Example I, 2c and the nucleic acid molecule encoding the BoNT/A receptor will be cloned using polymerase chain reaction (PCR) method. Genomic DNA from the C7 cell line will be isolated by an alkaline lysis procedure and will be amplified in PCR reactions using the ADVANTAGE[®] Genomic PCR kit (BD Biosciences Clontech, Palo Alto, Calif.) and the following two oligonucleotides 5'-AGCCCTCACTCCT-TCTCTAG-3' (SEQ ID NO: 29) and 5'-ACCTACAG-GTGGGGTCTTTC ATTCCC-3' (SEQ ID NO: 30). Reactions will be incubated at 95° C. for 1 minute, followed by 25 cycles at 68° C. for 30 seconds and 95° C. for 30 seconds, followed by 1 cycle at 68° C. for 6 minutes and final incubation at 4° C. The resulting PCR product will be purified from the PCR reaction by the QIAquick Gel Extraction Kit (QIAGEN, Inc., Valencia, Calif.), and will be subjected to a second PCR amplification. The oligonucleotides used in the second PCR will be nested primers designed to anneal to sequences found within the PCR product originally purified, and will have the following nucleotide sequences: 5'-CCCTGGGTCAAGCCCTTGTACACC-3' (SEQ ID NO: 31) and 5'-TGCCAAACCTACA GGTGGGGTCTT-3' (SEQ ID NO: 32). The resulting nested DNA product will be subcloned into a pTOPO[®]-XL vector using the TOPO[®] TA cloning method (Invitrogen, Inc., Carlsbad, Calif.). The ligation mixture will be transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, Inc., Carlsbad, Calif.) using a heat shock method, will be plated on 1.5% Luria-Bertani agar plates (pH 7.0) containing 100 μ g/mL of Ampicillin, and will be placed in a 37° C. incubator for overnight growth. Ampicillin-resistant colonies will be analyzed using an alkaline lysis plasmid mini-preparation procedure and candidate receptor constructs will be screened by restriction endonuclease mapping to determine the presence and orientation of the correct insert fragment. Cultures containing the desired expression construct will be used to inoculate 1 L baffled flasks containing 200 mL of Luria-Bertani media containing 100 μ g/mL of Ampicillin and will be placed in a 37° C. incubator, shaking at 250 rpm, for overnight growth. Purified plasmid DNA corresponding to an expression construct will be isolated using the QIAGEN Maxi-prep method (QIAGEN, Inc., Valencia, Calif.) and will be sequenced to verify that the correct expression construct was made (service contract with Sequetech Corp., Mountain View, Calif.). This cloning strat-

EMEM, supplemented with 10% FBS, 2 mM glutamine (Invitrogen, Inc, Carlsbad, Calif.), 1 mM sodium pyruvate (Invitrogen, Inc, Carlsbad, Calif.), 1.5 g/L sodium bicarbonate and 1×MEM non-essential amino acids solution (Invitrogen, Inc, Carlsbad, Calif.), and grown in a 37° C. incubator under 5% carbon dioxide until the cells reached a density of about 5×10⁵ cells/ml. The Neuro-2A cells were harvested by detaching the cells with a trypsin treatment, transferring the cells to 15 ml tubes, and centrifuging the cells at 5,000×g at 4° C. for 10 min. The cell pellet is washed three times with 9 mL of Tris-buffered saline, and then divided into aliquots of 4×10⁸ cells. Each aliquot of cells is suspended in 12 mL cold Tris-buffered saline for a final density of 2×10⁷ cells/mL, and placed on ice for 15 minutes. To one aliquot of cell suspension, 1 mL of PURE-A-SBED is added, final concentration is approximately 100 ug PURE A (33 nM). To a second cell aliquot, sulfo-SBED only is added and serves as a control for false positives. Both Neuro-2 cell suspensions were incubated at 4° C. for two hours in a secondary container using a shaking apparatus and then each cell solution is distributed in 13 aliquots of 1.0 mL. These aliquots were exposed to ultraviolet radiation (365 nm) at 4° C. for 15 minutes.

The cells were centrifugation at 5,000×g at 4° C. for 15 minutes and washed once with 1 mL cold Tris-buffered saline. Washed cells were lysed in 0.5 ml of lysis buffer containing 50 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 6.8, 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10% glycerol, 1% (v/v) Triton-X® 100 (4-octylphenol polyethoxylate) and suitable protease inhibitors, with rotation overnight at 4° C. Lysed cells were centrifuged at 5,000 rpm at 4° C. for 10 min to eliminate debris, the supernatants were transferred to fresh siliconized tubes and 0.05 mL of avidin-beads were added to the cleared supernatants. This mixture was incubated at 4° C. for 3 hours. The avidin beads were then washed twice by centrifuging at 1000×g at 4° C. for 10 min to pellet beads, decanting the supernatant, adding 0.5 mL lysis buffer and incubating the solution at 4° C. for 10 minutes. The avidin beads were then washed twice with 0.5 mL phosphate-buffered saline (pH 7.4). Approximately 100 μL of SDS-PAGE loading buffer was added to the washed, pelleted avidin beads and boiled for 10 minutes. A 40 μL aliquot was then subjected to MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen, Inc, Carlsbad, Calif.) under non-denaturing and denaturing, reducing conditions. FIG. 10a shows an approximately 250 kDa protein in non-reducing gels which represents the intact cross-linking reagent PURE-A-SBED toxin bound to the putative BoNT/A receptor. Same samples run under denaturing conditions and reveals an approximately 100 kDa protein was co-purified with PURE-A-SBED.

To determine the identity of the BoNT/A receptor isolated from the cross-linking experiments, western blot analysis was performed using antibodies to the cytoplasmic region of the polypeptides FGF 1 receptor (FGFR1), FGF 2 receptor (FGFR2), FGF 3 receptor (FGFR3) and FGF 4 receptor (FGFR4). Approximately 40 μL aliquots of the precipitated receptor-PureA complex, obtained as described above in Example II, 2, were separated by MOPS polyacrylamide gel electrophoresis using NuPAGE® Novex 4-12% Bis-Tris precast polyacrylamide gels (Invitrogen, Inc, Carlsbad, Calif.) under non-reducing and denaturing, reducing conditions. Separated peptides were transferred from the gel onto polyvinylidene fluoride (PVDF) membranes (Invitrogen, Inc, Carlsbad, Calif.) by Western blotting using a Trans-Blot® SD

semi-dry electrophoretic transfer cell apparatus (Bio-Rad Laboratories, Hercules, Calif.). PVDF membranes were blocked by incubating at room temperature for 2 hours in a solution containing 25 mM Tris-Buffered Saline (25 mM 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloric acid (Tris-HCl) (pH 7.4), 137 mM sodium chloride, 2.7 mM potassium chloride), 0.1% TWEEN-20®, polyoxyethylene (20) sorbitan monolaureate, 2% bovine serum albumin, 5% nonfat dry milk. Blocked membranes were incubated at 4° C. for overnight in Tris-Buffered Saline TWEEN-20® (25 mM Tris-Buffered Saline, 0.1% TWEEN-20, polyoxyethylene (20) sorbitan monolaureate) containing one of the following primary antibody solutions: 1) a 1:1000 dilution of rabbit polyclonal anti-FGFR1 antiserum (Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.); 2) a 1:1000 dilution of goat polyclonal anti-FGFR2 antiserum (Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.); 3) a 1:1000 dilution of rabbit polyclonal anti-FGFR3 (C15) antiserum (Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.); or 4) a 1:1000 dilution of goat polyclonal anti-FGFR4 antiserum (Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.). Primary antibody probed blots were washed three times for 15 minutes each time in Tris-Buffered Saline TWEEN-20®. Washed membranes were incubated at room temperature for 2 hours in Tris-Buffered Saline TWEEN-20® containing either a 1:20,000 dilution of goat polyclonal anti-rabbit immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (HRP; Pierce Biotechnology, Inc., Rockford, Ill.) as a secondary antibody for the FGFR1 and FGFR3 blots or a 1:20,000 dilution of rabbit polyclonal anti-goat immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (HRP; Pierce Biotechnology, Inc., Rockford, Ill.) for the FGFR2 and FGFR4 blots. Secondary antibody-probed blots were washed three times for 15 minutes each time in Tris-Buffered Saline TWEEN-20®. Signal detection of the labeled BoNT/A SNAP25₁₉₇-cleavage product was visualized using the ECL Plus™ Western Blot Detection System (Amersham Biosciences, Piscataway, N.J.) and the membrane was imaged and cleavage product quantitated with a Typhoon 9410 Variable Mode Imager and Imager Analysis software (Amersham Biosciences, Piscataway, N.J.). The choice of pixel size (100 to 200 pixels) and PMT voltage settings (350 to 600, normally 400) depended on the individual blot. A band was detected in 45 toxin-receptor sample probed with anti-FGFR3 antiserum of approximately 97 kDa that is consistent with the size of FGFR3, indicating that FGFR3 is a BoNT/A receptor (see FIG. 10b).

3. Identification of BoNT/A Receptor from Various Cells

Several cell lines responsive to BoNT/A uptake were probed with antibodies raised against FGFR1, FGFR2, FGFR3 and FGFR4 in order to determine which FGFRs these cell lines express. In addition, cells from the BoNT/A unresponsive HIT-T15 wild-type cell line and the BoNT/A responsive HIT-T15 isolate C7 cell line, as described above in Example I, 2c and 2d, were examined.

To determine the presence of FGFRs in cell lines responsive to BoNT/A exposure, cells were grown, harvested and lysed as described above in Example II, 1a, 1b or 2c and 40 μL aliquots were subjected to Western blot analysis as described above in Example II, 2. These results indicate that the BoNT/A responsive cell lines Neuro-2A, SH-SY5Y and HIT-T15-C7 all express FGFR3, while the BoNT/A unresponsive wild-type HIT-T15 does not (see FIG. 11). The data also from the revealed that FGFR2 and FGFR4 were not detected in any of the cell lines tested, while FGFR1 was present in all cell

lines tested, including wild-type HIT-T15 cells that are unresponsive to BoNT/A exposure (see FIG. 11).

4. Competitive Competition Assays

To corroborate that BoNT/A toxin enters Neuro-2A cells through the FGFR3 we performed a competition experiment with PURE-A and analyzed the responsiveness of tested using the SNAP-25 cleavage assay, as described above in Example I, 1b. If BoNT/A and an FGFR3 ligand bind to the same receptor, then increasing amounts of FGF ligand should result in decreased responsiveness of a cell to BoNT/A exposure. However, if BoNT/A and an FGFR3 ligand bind to the different receptors, then increasing amounts of FGF ligand should have no effect of the responsiveness of a cell to BoNT/A exposure. Table 1, which Applicants do not claim is a complete tabulation of FGF receptors and species, shows certain members of the family of FGFRs and their known ligands and tissue distribution.

To determine whether ligands for FGFR3 can competitively compete with BoNT/A for binding to FGFR3, about 5×10^5 Neuro-2A cells were plated in individual wells of a 6-well, poly-D-lysine/Laminin coated, tissue culture plates containing 3 mL of EMEM, supplemented with 2 mM glutamine (Invitrogen, Inc, Carlsbad, Calif.), 1 mM sodium pyruvate (Invitrogen, Inc, Carlsbad, Calif.), 1.5 g/L sodium bicarbonate and 1×MEM non-essential amino acids solution (Invitrogen, Inc, Carlsbad, Calif.), and grown in a 37° C. incubator under 5% carbon dioxide until the cells reached confluence. Approximately 5 nM PURE-A (Metabiologics, Inc., Madison, Wis.) was added in conjunction with FGF1, FGF2 or both FGF1 and FGF2 at different concentrations (0 nM, 0.1 nM, 1 nM, 5 nM, 50 nM, 200 nM) in the culture medium containing the cells and incubated for at 37° C. for approximately 10 minutes Cells were collected in 15 ml tubes, washed once with 1 ml of phosphate-buffered saline, pH 7.4, and then transferred to 1.5 ml microcentrifuge tubes. Cells were lysed in 0.5 ml of lysis buffer containing 50 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 6.8, 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10% glycerol and 1% (v/v) Triton-X® 100 (4-octylphenol polyethoxylate), with rotation for 1 hour at 4° C. Lysed cells were centrifuged at 5000 rpm for 10 min at 4° C. to eliminate debris and the supernatants were transferred to fresh siliconized tubes. Protein concentrations were measured by Bradford's method and resuspended in 1×SDS sample buffer at 1 mg/ml or higher concentration.

The presence of a BoNT/A SNAP25₁₉₇-cleavage product was determined by Western blot analysis as described above in Example II, 1a, with the exception that blocked PVDF membranes will be incubated in a primary antibody solution containing a 1:50,000 dilution of mouse monoclonal anti-SNAP-25 antibody (SMI-81; Sternberger Monoclonals, Lutherville, Md.) rather than the rabbit polyclonal anti-SNAP25 antiserum pAb anti-SNAP25197 #1 and a secondary antibody solution containing a 1:20,000 dilution of goat polyclonal anti-mouse immunoglobulin G, heavy and light chains (IgG, H+L) antibody conjugated to horseradish peroxidase (HRP; Pierce Biotechnology, Inc., Rockford, Ill.) rather than the goat polyclonal anti-rabbit IgG-HRP antibody in order to detect both the uncleaved SNAP-25 substrate and BoNT/A SNAP25₁₉₇-cleavage product. An increasing amount an increasing amount of FGF ligands, indicating these FGF1 and FGF2 compete for the same receptor as BoNT/A and further confirming that FGFR3 is a BoNT/A receptor (see FIG. 12).

Example III

A fusion protein comprising the C terminal portion of the heavy chain of BoNT/A and the light chain of BoNT/E is tested for its ability to selectively bind and intoxicate BoNT/A susceptible cells. A preparation comprising dilutions of the fusion protein is incubated with HIT-T15 insulinoma cells expressing exogenous FGFR3 in the presence of GT1b ganglioside. The ability of the fusion peptide to bind and enter the insulinoma cells is detected by detecting secretion of insulin in response to the presence of glucose, as described above in Example I, 1a. By contrast, insulin secretion is unaffected in cells not expressing FGFR3.

The results of this assay show that amount of insulin secreted into the culture medium is decreased in a dose-dependent manner when the fusion protein is added to the culture medium. Western blots of cell lysates will show the conversion of full length SNAP-25 to the cleaved form typical of the proteolytic activity of the BoNT/E light chain protease. This assay therefore is useful in showing that the fusion peptide is able to bind and enter BoNT/A susceptible cells.

The same fusion protein is capable of intoxicating cells of the neuromuscular junction.

Example IV

A fusion protein comprising the receptor binding portion of an FGF species capable of binding FGFR3 (including FGF1, FGF2, FGF4 and FGF9) and the translocation domain and light chain of BoNT/E is tested for its ability to selectively bind and intoxicate BoNT/A susceptible cells. The assay is conducted as described in Example 1 above, with similar results; the detected cleaved SNAP-25 fragments are characteristic of BoNT/A intoxication.

Example V

BoNT/A, produced from fermentation of *Clostridium botulinum* is produced using standard fermentation techniques. Either or both the bulk preparation and purified, formulated versions of expressed toxin are tested for purity and activity as follows. A preparation comprising dilutions of the BoNT/A preparation is incubated with HIT-T15 insulinoma cells expressing exogenous FGFR3 in the presence of GT1b ganglioside. The ability of the toxin to bind and enter the insulinoma cells is detected by detecting secretion of insulin in response to the presence of glucose, as described above in Example I, 1a. The specific activity of the preparation can be calculated from the determined protein concentration and the activity of the preparation at various doses.

These data are submitted to the U.S. Food and Drug Administration by a pharmaceutical company as part of data demonstrating how BoNT/A is manufactured and tested. This information is considered by the FDA, who decides to permit the manufacture and sale of this lot of BoNT/A, and subsequent lots made and tested in a similar manner, as a therapeutic pharmaceutical product based in part on this bulk and/or formulation assay data.

The pharmaceutical comprising the BoNT/A is then offered for sale as a prescription medication.

Example VI

Same as Example V, however the polypeptide produced is the fusion neurotoxin of Example III, produced in *E. coli*. Both bulk and/or formulation lots of the fusion neurotoxin are tested as indicated above, the data submitted to the FDA, and